ASPEN LEAF MINER (*PHYLLOCNISTIS POPULIELLA*) OVIPOSITION SITE SELECTION MEDIATED BY EXPRESSION OF EXTRAFLORAL NECTARIES

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ASPEN LEAF MINER (*PHYLLOCNISTIS POPULIELLA*) OVIPOSITION SITE PREFERENCE MEDIATED BY EXPRESSION OF EXTRAFLORAL NECTARIES

A

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ABSTRACT

As herbivorous insects choose where to lay their eggs, maximizing larval performance (optimal oviposition) may be at odds with adult foraging (optimal foraging). Aspen leaf miners (*Phyllocnistis populiella*; ALM) choose between leaves with or without extrafloral nectaries (EFNs) as oviposition sites on quaking aspen (*Populus tremuloides* Michx.). Reduced ALM mining on leaves with EFNs may be linked to predators that defend leaves and feed at EFNs, and to increased concentrations of secondary leaf compounds. However, direct responses of adult ALM to EFN expression may also explain differences in ALM mining among leaves with and without EFNs. ALM might be less likely to oviposit on leaves with EFNs because these leaves provide poor conditions for larval development. With choice experiments and surveys of ALM oviposition, we examined oviposition site preference in relation to EFN expression. In choice experiments and field surveys adults preferred to oviposit on leaves without EFNs. Increased oviposition was also observed on shoots with high EFN frequencies, suggesting a combination of optimal foraging and optimal oviposition, and a role of EFNs at scales above the leaf level. Higher predation on leaves with EFNs likely exerts selective pressure for the observed ALM oviposition preference for non-EFN leaves.

KEY WORDS Aspen, *Populus tremuloides*, optimal foraging, optimal oviposition, Alaska

Table of Contents

Signature Page i
Title Page iii
Abstract v
Table of Contents vii
List of Figures viii
List of Tables ix
Acknowledgments xi
Introduction 1
Methods 9
Phyllocnistis populiella and Aspen EFNs 9
2-Way Choice 11
3-Way Choice 14
Long-Term Survey 16
Egg Tracking Survey 18
Results 21
2-Way Choice 21
3-Way Choice 21
Long-Term Survey 23
Egg Tracking Survey 27
Discussion 31
EFN-mediated oviposition site preference and egg predation 31
Intraspecific competition 35
Leaf Side 37
Predator Exclusion 38
Conclusion 38
References 40

List of Figures

Figure 1. Aspen leaves, EFNs, and visitors 10

Figure 2. Diagram of 2-Way Choice chamber with mated pair of ALM.....13

Figure 3. Glue applications of leaves in the 3-Way Choice experiment.....15

Figure 4. EFN frequency varies with leaf position 18

Figure 5. ALM oviposition on leaves with and without EFNs in 2-Way Choice trials....22

Figure 6. ALM oviposition on leaf types in the Long-Term Survey 24

Figure 7. Effect of shoot EFN frequency on the sum of eggs, scars and mines per leaf surface 25

Figure 8. The sum of eggs, scars and mines on leaf tops and leaf bottoms in the Long-Term Survey.....26

Figure 9. Oviposition on leaves with and without EFNs on ramets with crawling predators excluded versus control.....28

Figure 10. Oviposition on leaf tops and bottoms at varying shoot EFN frequencies 28

Figure 11. Egg removals on leaves with and without EFNs in the Egg Tracking Survey 29

Figure 12. Egg removals at varying shoot EFN frequencies in the Egg Tracking Survey 30

List of Tables

Table 1. Site locations and elevations for collection of experimental material and surveys 11

Table 2. Analysis of oviposition (eggs, scars, and mines) and egg removal (scars) in the Long-Term Survey data 23

Table 3. Analysis of oviposition (eggs) and egg removal (removals) in the Egg Tracking Survey 27

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[1] Dennis, R., P. Daok, and D. Wagner. In preparation for Ecological Entomology.

INTRODUCTION

Phytophagous insects appear to often choose oviposition sites based on factors that impact maternal and offspring fitness (Scheirs and De Bruyn 2002b, Refsnider and Janzen 2010). These choices are made at several scales from plant-level selection (Kuussaari and Singer 2000, Hanski and Singer 2001) to microsite selection within host individuals (*reviewed in* Thompson and Pellmyr 1991). The optimal oviposition hypothesis (also called the preference-performance hypothesis) predicts that females will prefer oviposition sites that maximize individual larval performance (Jaenike 1978, Thompson 1988, Mayhew 1997). However, for insects that forage as adults, searching for oviposition sites that confer the greatest larval fitness requires time and energy that could otherwise be invested in adult foraging (Thompson 1988, Mayhew 1997, Scheirs and De Bruyn 2002b, Janz et al. 2005, Gripenberg et al. 2010) and further egg production. In addition, the prolonged search time associated with optimal oviposition may increase the risk of maternal mortality. The optimal foraging hypothesis predicts oviposition behavior that maximizes maternal fecundity by optimizing adult nutrition (Jaenike 1986, Stephens and Krebs 1986, Scheirs et al. 2000, 2004, Janz et al. 2005, *but see* Pyke 1984). These oviposition strategies are mutually exclusive if high quality oviposition sites and feeding sites are physically separated, leaving females to compromise between optimal oviposition with suboptimal foraging versus optimal foraging with suboptimal oviposition. Oviposition site selection in European grass miners, for example, optimizes adult feeding location but has only weak positive effects on offspring development and

survival (Scheirs et al. 2000). However, in the pea leaf miner (*Liriomyza huidobrensis*), oviposition favors sites where larval development is optimized and adult foraging is suboptimal (Videla et al. 2012).

Support for optimal oviposition is strong (*see* Gripenberg et al. 2010). However, many others report no or negative correlation between adult oviposition site preference and offspring performance in a wide variety of insect genera (*reviewed in* Mayhew 1997), calling the ubiquity of optimal oviposition into question. Studies that integrate tests of optimal oviposition and optimal foraging have helped to explain variation in the relationship between oviposition site choice and offspring performance reported in previous studies (Scheirs et al. 2000, Mayhew 2001, Scheirs and Debruyn 2002a, Scheirs et al. 2004). Still, recent research is heavily biased toward tests of optimal oviposition, while optimal foraging is often invoked merely to explain weak or negative results (*see* Johnson et al. 2006, Nufio and Papaj 2004).

Implicit in the optimal oviposition and optimal foraging theories is the ability of females to detect the quality of oviposition sites or the presence of foraging sites. To locate and determine the quality of these sites, insects use visual, tactile, auditory, olfactory, and gustatory signals (Ramaswamy 1988, Almohamad et al. 2009), including those related to the presence of predators or competitors and adult food resources. Such signals can explain avoidance of foraging and oviposition sites that carry high predation risks for adults and later for eggs and larvae, as well as preference for those sites that optimize foraging and/or larval performance.

The presence of natural enemies or competitors can deter adult visitation to potential hosts (Freitas and Oliveira 1996, Goncalves-Souza et al. 2008). *Eunica* butterflies, for example, recognize predators by sight and avoid laying eggs near ants that are aggressive toward larvae (Sendoya et al. 2009). In *Oreina* leaf beetles, the presence of predators on high quality hosts leads to oviposition preference for hosts that support relatively low offspring performance even in the absence of predators (Ballabeni et al. 2001). Similarly, the presence of conspecific eggs and larvae can deter subsequent oviposition (Poirier and Borden 1991, Vasconcellos-Neto and Monteiro 1993, Ulmer et al. 2003, Sugiura et al. 2007, *reviewed in* Thompson and Pellmyr 1991). *Mechanitis lysimnia* butterflies, for example, detect conspecific eggs at potential oviposition sites and prefer ovipositing on egg-free hosts (Vasconcellos-Neto and Monteiro 1993).

In addition to signals derived directly from predators and competitors, plant-derived cues may lead to oviposition site preference or avoidance. These signposts can advertise risk of predation or competition whether or not predators or competitors are present, leading to oviposition avoidance based on optimal oviposition. For example, volatile organic compounds (VOCs) induced by damage to tobacco plant tissue simultaneously attract natural enemies and deter ovipositing herbivores due either to increased risk of intraspecific competition or predation (Kessler and Baldwin 2001).

Nectar is an important food source for many adult insects and may positively influence oviposition site selection based on foraging opportunities among and within host plants. For nectivorous pollinators like the Common Blue butterfly (*Polyommatus icarus*), the availability of floral nectar alone can drive oviposition site selection (Janz et

al. 2005). Extrafloral nectar can similarly affect oviposition site preference; some Lepidopteran herbivores of cotton prefer to oviposit on plants with extrafloral nectaries, at which adults feed, despite the attraction of predaceous ants to extrafloral nectar (Adjei-Maafo' and Wilson 1983).

Nectar might also serve as a warning sign to ovipositing herbivores indicating the possible attraction of natural enemies. Extrafloral nectaries (EFNs) are secretory glands located on plant leaves, stipules, stems, and external flower parts, and are commonly associated with ant-plant protection mutualisms (Bentley 1977, Koptur 1992). Extrafloral nectar often elicits defense of leaf tissue by attracting ants and other predators that harass or remove herbivores (*reviewed in* Bentley 1977, Koptur 1992, Bronstein 1998, Heil and McKey 2003, Mondor and Addicott 2003, Huntzinger et al. 2004, Ness et al. 2009). Therefore, herbivores might avoid ovipositing near EFNs due to their potential to attract natural enemies. However, the occurrence of plant populations where EFN-mediated predation does not lead to a net reduction in herbivory (*reviewed in* Becerra and Venable, 1985) suggests that other mechanisms for the maintenance of this plant trait exist. For example, EFNs may not only attract prospective mutualists of the plant and warn of the risk of predation but also convey leaf quality through their association with other leaf traits.

We used a system with natural variation in EFN expression to examine whether EFNs influence oviposition site selection by a specialist herbivore. Adult aspen leaf miner moths (*Phyllocnistis populiella* Cham., Lepidoptera: Gracillariidae, hereafter ALM) feed on quaking aspen (*Populus tremuloides*) EF nectar throughout the oviposition

period, and ALM larvae feed within aspen leaves. Thus, the presence of aspen EFNs has the potential to impact both adult foraging and offspring performance. Further, aspen express EFNs on a subset of their leaves at varying frequency thereby presenting ovipositing females with a dichotomous choice between leaves with and without EFNs as well as choices at larger scales among shoots and ramets with diverse EFN frequencies.

Aspen leaves with EFNs incur less ALM damage than leaves without EFNs (Doak et al. 2007, Mortensen et al. 2011). In addition to EFN-mediated defense at the leaf scale, the attraction of ants and other predators to EFNs provide a "diffuse defense" at the ramet scale by protecting both EFN and non-EFN leaves on ramets with a high frequency of EFN leaves (Mortensen et al. 2011). However, reduced ALM mining on aspen leaves with EFNs persists when crawling predators are excluded (Mortensen et al. 2011). This suggests that EFNs may influence oviposition behavior of adult ALM even in the absence of predators.

The potential influence of aspen EFNs on oviposition site choice is complicated by the different relationship that EFNs have with adult foraging and larval performance. Optimally, a female should lay a full complement of high quality eggs at high quality oviposition sites while maximizing energetic intake for continued survival and egg production. ALM adults appear to feed exclusively at aspen EFNs (personal observation). Foraging economics that maximize EF nectar intake and minimize travel time may therefore result in increased egg loads and oviposition on or near foraging sites (EFN-leaves). However, these leaves may be relatively poor oviposition sites, as they are visited by ALM predators and parasitoids that also feed on EF nectar. In addition, EFN

leaves have higher concentrations of phenolic glycosides associated with decreased ALM mining damage (Young et al. 2010). The costs and benefits of ovipositing on leaves near foraging opportunities versus those best suited for larval development may constitute a trade-off between egg placement based on optimal foraging and optimal oviposition.

The characteristics of a leaf's neighborhood, as well as the leaf itself, seem to be important to ovipositing moths. Independent of leaf EFNs, the extent of ALM mining damage is reduced on ramets with high EFN frequencies (Mortensen et al. 2011). Furthermore, the proportion of EFN leaves on an aspen ramet interacts with leaf-level EFN expression to reduce the number of eggs laid on EFN leaves at low and average EFN frequencies (Mortensen et al. 2013). These results suggest that oviposition site selection might depend upon leaf neighborhood as well as individual leaf quality, including leaf physiology and appeal to predators. For instance, preferential oviposition on leaves of shoots with high EFN frequencies would be consistent with optimal foraging, whereas oviposition preference for shoots with low EFN frequencies would be consistent with optimal oviposition. Shoots with low EFN frequencies would be preferred oviposition sites if larval development in neighborhoods with low predation is a priority.

We examined ALM oviposition site choice and egg predation in relation to aspen EFN expression during a period of high ALM density. If ovipositing ALM detect EFNs, they might avoid leaves that bear them, preferring to oviposit in locations less likely to attract predators and/or with lower concentrations of secondary leaf compounds. On the other hand, attraction of adult ALM to foraging opportunities at EFNs might result in higher oviposition not only on leaves bearing EFNs, but also shoots, ramets, and even

stands with high EFN frequencies. Based on lower mining damage on EFN leaves and lower mining and oviposition on shoots and ramets with high EFN frequencies (Doak et al. 2007, Mortensen et al. 2011, Mortensen 2013), we hypothesized that aspen EFNs negatively affect leaf-level ALM oviposition site preference at both the leaf and shoot scales. We also looked at whether patterns of egg predation suggest differential risk to ALM eggs with relation to EFN expression.

We used a combination of choice experiments and field surveys to test the effects of EFN expression on ALM oviposition. Choice experiments were conducted to test oviposition site preference for leaves with and without EFNs and to examine the separate impacts of EFN expression and nectar availability. Six years of field survey data were used to examine patterns of oviposition and egg predation in relationship to EFN expression. An additional single-season field survey was used to examine patterns of oviposition and egg predation during the first days of the oviposition period when egg densities (i.e. competition) were low.

METHODS

***Phyllocnistis populiella* and Aspen EFNs.** In interior Alaska, ALM adults emerge from overwintering sites in the leaf litter in late April and early May. Adults seek out water and extrafloral nectar, which seem to be the only sources, in addition to spermatophores, used by adult moths to sustain foraging, mating, and oviposition activity (Condrashoff, 1964, personal observation). During aspen bud break, males and females aggregate on tree trunks, leaf bottoms, and other shaded surfaces to mate. Mating bouts of approximately 20-30 minutes (Condrashoff 1964) occur throughout the oviposition period from mid-May to early June in Alaska.

Females deposit eggs singly onto the upper or lower surfaces of young leaves. These eggs sink into the leaf tissue where larvae hatch and access the epidermis. Alternatively, eggs may be preyed upon by ants, mites, or other egg predators, or fail to hatch altogether. Eggs that are removed after beginning to sink into the leaf tissue leave a distinctive depression (hereafter scar) on the leaf surface. Larvae hatched from the surviving eggs mine the single layer of epidermal cells beneath the cuticle (Figure 1c) for two to three weeks (Condrashoff 1964). At high ALM densities, multiple eggs are regularly deposited on a leaf, and encounters between the mining larvae often result in mortality (Condrashoff 1964, Doak and Wagner unpublished data). Successful larvae then pupate in folds along the leaf edge, and eclose as adults in late June and early July (Condrashoff 1964). These adults will then overwinter in the leaf litter, then emerge, mate, and oviposit on aspen leaves the following spring.

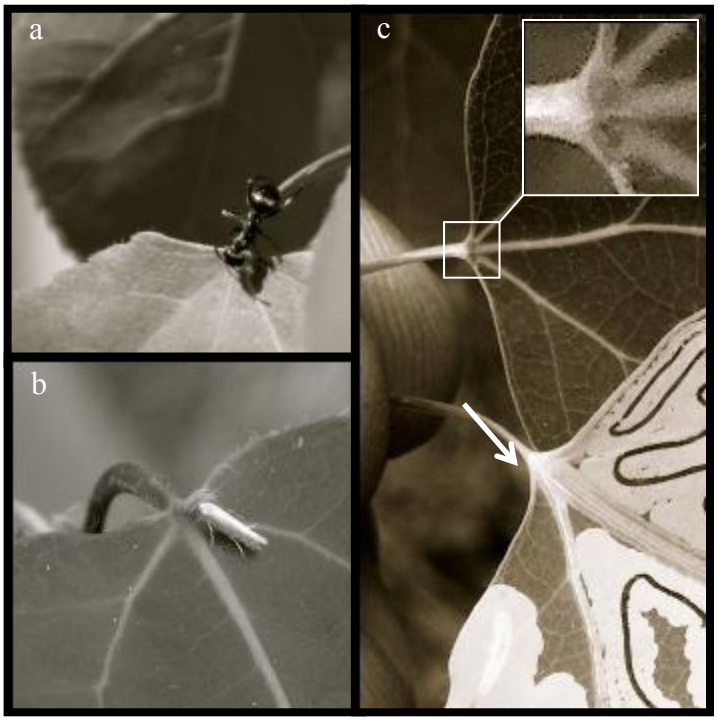

Figure 1. Aspen leaves, EFNs, and visitors. Ants (a) and adult ALM (b) nectar at aspen EFNs (inset). ALM larvae (arrow) chew serpentine mines on either surface of aspen leaves.

In quaking aspen (*Populus tremuloides*) EFN expression is variable within and among ramets and genets (Wooley et al. 2007). In preformed leaves, which overwinter as leaf primordia, EFN expression is high at proximal positions and decreases distally along the shoot (Doak et al. 2007). EFN expression is also high on neoformed leaves that grow as shoots extend throughout the growing season (Doak et al. 2007). In several species, including quaking aspen, EFN expression is induced by herbivory (Mondor and Addicott 2003, Huntzinger 2004, Wooley et al. 2007), and this is likely to contribute to gene-by-environment variation in EFN frequency among ramets and genets.

2-Way Choice. In the summer of 2009, we conducted choice experiments to test whether female ALM preferentially oviposit on aspen leaves with or without EFNs. Before bud break, we bagged aspen shoots on ramets <2m in height at seven sites near Fairbanks, Alaska (USA, 64°49'N, 147°59'W, Table 1) with fine mesh to prevent ALM oviposition. After bud break we cut bagged shoots as needed until our requirements for leaves exceeded the availability of bagged shoots. When unbagged shoots were collected, we selected leaves without ALM eggs when possible; when eggless leaves were not available, eggs were carefully removed from leaves prior to use in experiments.

Table 1. **Site locations and elevations for collection of experimental material and surveys.** Sites were selected for a combination of uses; shoots and ALM collection for choice experiments (ALM, shoots), Long-Term Survey (LTS), and/or the Egg Tracking Survey (ERTS).

Site	Latitude	Longitude	Elevation (m)	Use
BNZ	64°42'36.00"N	148°19'35.28"W	231	LTS
DO	64°51'27.28"N	147°50'04.47"W	178	ALM/shoots
ED	64°52'36.48"N	148°03'51.36"W	719	ALM/shoots/egg
GH	64°51'27.06"N	147°50'24.30"W	187	shoots/ LTS/ERTS
MF	64°53'02.58"N	147°54'19.31"W	213	ALM/shoots
RF	64°51'31.37"N	147°52'10.25"W	270	Shoots
RP	64°49'03.60"N	147°58'24.00"W	247	ALM/LTS
SD	64°51'31.41"N	147°51'24.50"W	189	shoots/ERTS
WR	64°51'26.14"N	147°51'31.87"W	185	ALM/shoots/LTS

ALM moths were collected from aspen leaves, trunks of mature trees, and artificial surfaces. We ensured collection of gravid females by capturing mating pairs. Pairs of moths were placed in vials containing a single young leaf to provide moisture, and were stored at 2.5°C for use in choice trials. Each choice arena (Figure 2) was fashioned from a 475ml clear plastic food container with the top and bottom replaced

with fine mesh. We cut a slot on each side of the choice arena to allow insertion of leaves. Two shoots collected from the same site were placed in water-filled floral tubes glued to either side of the container. An EFN leaf was selected on one shoot, and a non EFN leaf was selected on the other. These leaves were inserted into the slots to approximately half way between the leaf-petiole junction and the base of the petiole. The EFN and non EFN leaves each trial were from the same site, and selected so that they were of similar size, phenology and leaf position. For each of 167 trials, we introduced one mated pair of ALM and allowed 4-8 days for oviposition while recording twice daily the number of eggs deposited on each leaf.

When possible, we recorded the leaf on which the first egg was laid. Trials were terminated if both ALM died or when leaves showed signs of wilting. During morning and evening hours when ALM adults are normally active, chambers were placed in a forested outdoor environment to approximate natural light and temperature conditions. ALM are inactive during peak daylight hours; during this period chambers were moved indoors to avoid direct sunlight and subsequent desiccation and overheating of moths.

To examine whether moths displayed a preference for EFN vs. non-EFN leaves, we compared the observed ratio of leaves chosen for the first oviposition event to an expected ratio of 1:1 using a Chi-square test. The first oviposition in each trial represents choice in the absence of competition (i.e., previously laid eggs). We also ran this analysis using the total number of eggs laid to test for preference between leaf types when previously laid eggs were present on some leaves. As a test of the effects of intraspecific competition in the context of EFN-mediated preference, we compared the number of trials in which eggs were dispersed over two leaves to trials with multiple eggs on one leaf using a Chi-square test.

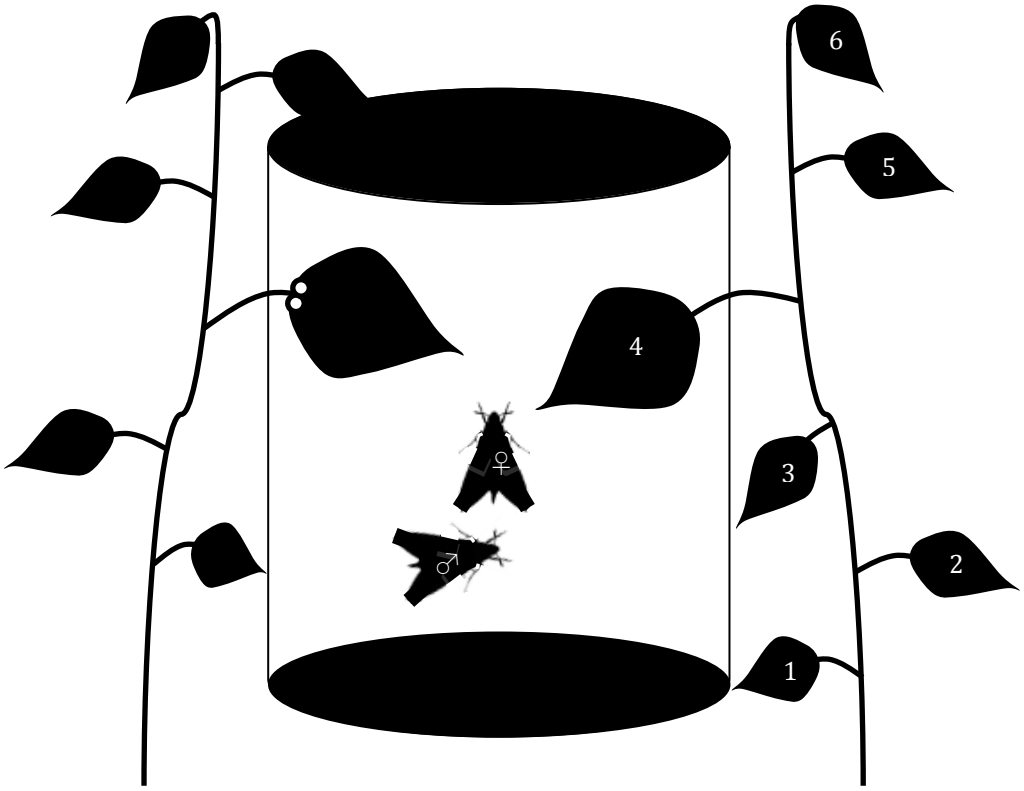

Figure 2. Diagram of 2-Way Choice chamber with mated pair of ALM. Numbers reflect leaf position, and white dots represent EFNs. Moths and leaves not to scale.

3-Way Choice. In 2010 we conducted a 3-way choice experiment to separate the effects of EFN-associated leaf traits (e.g. phenolic concentration) and nectar availability on ALM oviposition. Each female ALM was presented with three leaves, one with EFNs, one without EFNs, and another with EFNs occluded by glue (hereafter EFN, non-EFN, EFN-occluded leaves, respectively, Figure 3). We occluded EFNs on the third leaf so that females could choose to oviposit where nectar is unavailable and leaf quality is assumed to be low relative to leaves without EFNs. Higher oviposition on EFN-occluded and non-EFN leaves would suggest that the lack of extrafloral nectar, and not EFN-associated leaf traits, drives preference. Higher oviposition on leaves with exposed EFNs relative to the other two leaf types would suggest optimal foraging. Higher oviposition on non-EFN leaves would suggest optimal oviposition based on a lack of EFN-associated leaf traits on non-EFN leaves. Equally high oviposition on the two leaves with EFNs relative to the leaf without EFNs would suggest that leaf characteristics associated with EFN expression influence oviposition site choice.

We modified the design of the arenas from the 2-way experiments by adding a third slot to accommodate the additional leaf. On EFN-occluded leaves a drop of non-toxic glue (Aleene's OK to Wash It Glue, Duncan Enterprises, Fresno, CA, USA) was placed on top of the EFNs. To control for possible effects of the glue, we placed glue at the leaf-petiole junction on non-EFN leaves and adjacent to the EFNs on EFN leaves (Figure 3). Moths and leaves for use in these trials were collected in the same way as in the 2-way choice trials with all shoots within a trial were from the same site. In the 3-way choice trials we supplemented ALM diet with a wick inserted into a 1.5mL

microcentrifuge tube containing approximately 1mL of 10% sucrose solution. Trial durations and data collection were similar to the 2-way choice trials.

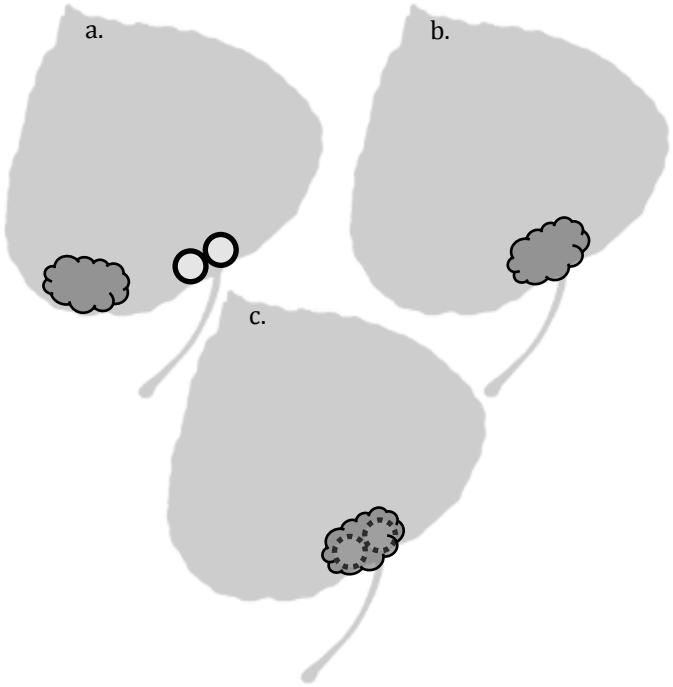

Figure 3. Glue applications on leaves in the 3-Way Choice experiment. In each trial a drop of glue was placed adjacent to EFNs, at the leaf-petiole junction, or over EFNs on the EFN leaf (a), non-EFN (b), and EFNs occluded (c) respectively.

We examined whether the oviposition site of the first egg in each successful trial was related to EFN availability by comparing the number of first eggs laid on EFN, non-EFN, and EFN-occluded leaves to ratios expected under no preference. We made a three-way comparison, using a G-test, to test for preferences among the three leaf types. We then used pair-wise comparisons to test whether the location of the first oviposition was related to: 1) nectar availability (EFN vs. EFN-occluded), and 2) other, non-nectar leaf traits associated with EFN expression (non-EFN vs. EFN-occluded).

Long-Term Survey. From 2006 to 2012 we conducted early season surveys of ALM eggs and scars to assess patterns of ALM oviposition and egg predation on leaves with and without EFNs. We established four sites near Fairbanks, Alaska (USA, 64°49'N, 147°59'W, Table 1) that were typically located on the margins of mature aspen stands. The likelihood of genetic overlap among sites was reduced by establishing sites in stands separated by another forest type and at least four kilometers.

Surveys were conducted in late May, near the end of the oviposition period. We sampled a single shoot from each of approximately 30 haphazardly chosen ramets (<2 m height) at each site. On each leaf, we counted the number of ALM eggs, egg scars, and newly initiated mines per leaf side (top and bottom tallied separately). Leaves were surveyed distally along each shoot so that leaf position one is proximal to two, etc. We summed eggs, scars, and initiated mines to estimate the total number of eggs laid on each leaf side. Given that some oviposition likely occurred after our surveys and some egg predation may have occurred prior to scar formation, our counts represent lower bounds on egg density.

To test for the influence of EFN expression on ALM oviposition we used a generalized linear mixed model (PROC GLIMMIX; SAS Institute Inc., Cary, North Carolina, USA) of the Long-Term Survey data using a Poisson distribution. We tested the response of the sum of eggs, scars, and mines to the fixed effects of leaf EFNs (presence/absence), shoot EFN frequency (percent of all leaf positions with EFNs per shoot), leaf side (top/bottom), as well as the interactions of leaf EFNs by shoot EFN

frequency and leaf side by shoot EFN frequency, which might reveal effects of EFNs exclusivity to leaf tops at varying EFN frequencies. Leaf EFNs by leaf side was excluded from this model because running the model with it included resulted in non-convergence. We also included the random grouping effects of year, site nested within year, shoot nested within site and year, and leaf nested within year, site and shoot.

We used logistic regression to test for the effects of EFNs on egg removal as indicated by the presence of scars. We modeled egg removal events by including two possible outcomes of egg deposition as the response: egg intact or egg scar. The fixed and random effects were the same as in the previous model with the addition of the leaf EFNs by leaf side interaction. This model was centered so that scar estimates were calculated based on mean shoot EFN frequency.

EFN expression changes predictably with leaf position, with decreased EFN expression at distal positions (Doak et al. 2007, Young et al. 2010). Because we were interested in positions with sufficient variation in EFN expression to offer choice to ovipositing females we restricted survey analyses to positions at which EFNs occurred on 25 to 75 percent of leaves. This led to the exclusion of positions 1 and 2 where EFN expression is nearly ubiquitous (Fig. 4). In this way, we included only those leaf positions with good representation of both leaf types. We also removed positions >7 because few shoots have leaves at higher positions during ALM oviposition. We excluded any leaves with more than 10% missing leaf area as we could not account for incidental removal of eggs by chewing insects (e.g., beetles). Post-hoc t-tests with Tukey adjustments for multiple comparisons were used to examine significant interaction terms.

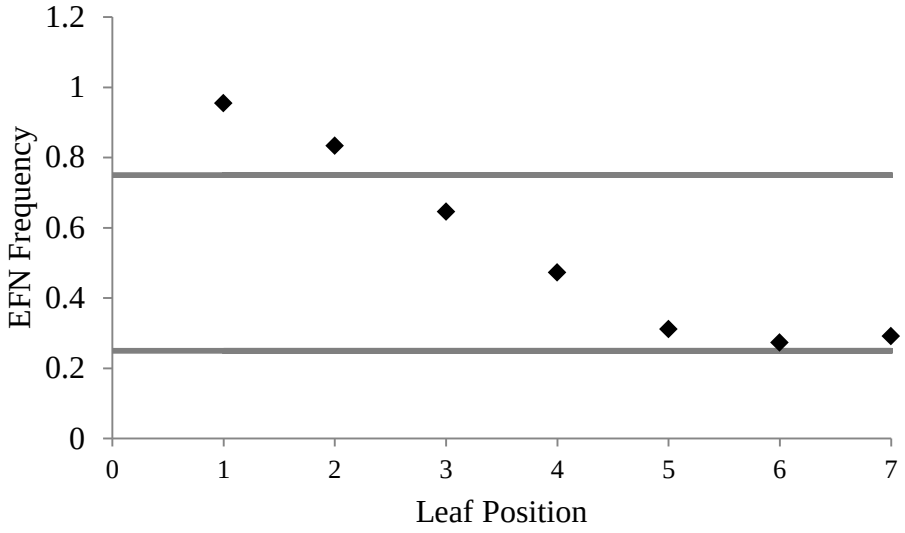

Figure 4. EFN frequency varies with leaf position. The bounds of tolerance (25% presence or absence) for inclusion in the models of ALM oviposition and egg scars based on the 2006-2012 Long-Term Survey are represented by solid lines. Leaf positions > 7 are rarely available during oviposition and were therefore excluded from the model.

Egg Tracking Survey. In order to test the effects of predation on ALM oviposition in greater detail, and to capture oviposition site preference at the beginning of the oviposition period when conspecific egg densities are low, we carried out an additional survey at two sites in Fairbanks, AK (Table 1) from May 19 to May 30, 2011. By repeatedly surveying leaves early in the season and tracking the fate of individual eggs we were able to account for egg removals or loss that did not produce scars. We included an exclusion treatment to test whether crawling predator access affected ALM oviposition site selection or egg removal. We selected ten ramets (<2m ht) at each of two sites, and before budbreak, we applied a band of sticky gel (Tangle-Trap; Tanglefoot®, Grand Rapids, MI, USA) around the main stem of five randomly chosen ramets to

exclude crawling predators. Three shoots on each ramet were haphazardly selected and tags affixed to the internode distal to each shoot to avoid possible effects of tag contact with crawling predators on non-exclusion ramets. Leaf positions 1-5 were surveyed daily from May 19-24 and every other day from May 25-30. Leaf positions > 5 were available for oviposition only briefly during the survey and were therefore excluded from the study. Each egg was assigned an identification number and plotted on a leaf diagram allowing us to track individual egg fate over the study period. For each leaf we recorded the number of EFNs, and for each egg we recorded removal with or without a scar or mine initiation for hatched eggs.

We tested the response of ALM oviposition and egg removal to EFN expression and crawling predator exclusion using generalized linear mixed models and logistic regression, respectively (PROC GLIMMIX; SAS Institute Inc., Cary, North Carolina, USA). In the Long-Term Survey we analyzed leaf positions 3-7 in order to address leaf-level preference where variation in EFN expression represents choice between leaves with and without EFNs. In the Egg Tracking Survey, we included positions 1-5 to address oviposition and predation at all leaf positions available during early oviposition. EFNs are expressed on over 80% of leaves at positions 1 and 2 (Figure 4). By including these positions we hypothesized that EFN expression at the leaf and shoot scales is associated with a reduction in ALM oviposition even when variability in EFN expression related to leaf position is not controlled. We separately modeled the total number of eggs and egg removals per leaf side with fixed effects of leaf EFNs, shoot EFNs frequency, predator exclusion, leaf side, and all two-way interactions using Poisson and binomial

distributions, respectively. The interaction terms for this and the following model differ slightly from those in the Long-Term Survey with the addition of a treatment group in the Egg Tracking Survey. We also had to simplify the model by removing the shoot EFN frequency by leaf side interaction to allow it to run in SAS. In the model of egg removals we included two possible outcomes of egg deposition as the response to the fixed effects: removal or no removal. To control for nestedness we included the random effects of site, ramet within site, and shoot within ramet within site in both models. In the case of significant interactions, we ran post-hoc comparisons with Tukey adjustments. Incomplete data led to the exclusion of nine shoots from the analyses; however each ramet was represented by at least two shoots. In addition, a single leaf side with 6 eggs was excluded as an outlier.

RESULTS

2-Way Choice. ALM moths laid at least one egg in 48 of 167 choice trials. Of these, 25 trials resulted in a single oviposition. In 11 of the 23 trials in which multiple eggs were laid we were able to determine whether the first egg was laid on the EFN vs. non-EFN leaf. Mean time to first oviposition was approximately one day from the time that moths were released into the choice arena, with a maximum of four days. The first egg in each trial was laid more often on a non-EFN leaf than was expected under no preference ($\chi^2 = 6.429$, $P = 0.011$, n = 36; Figure 5a). When considering all eggs, more were laid on non-EFN leaves, but there was not a significant difference between the two leaf types ($\chi^2 = 1.988$ $P = 0.159$, n = 85 eggs; Figure 5b). In trials with multiple eggs, oviposition eggs were laid more often on both leaves than on one leaf than would be expected by chance ($\chi^2 = 0.049$, $P < 0.001$, n = 21).

3-Way Choice. Oviposition occurred in 47 of 135 trials. Mean time to oviposition was approximately two days. Females laid 11 first eggs on EFN leaves, 16 first eggs on non-EFN leaves, and 20 first eggs on EFN-occluded leaves. Analyses considering the first eggs laid provided no evidence for differences in oviposition preference among the three leaf types ($G = 2.66$, $P > 0.05$, n = 47).

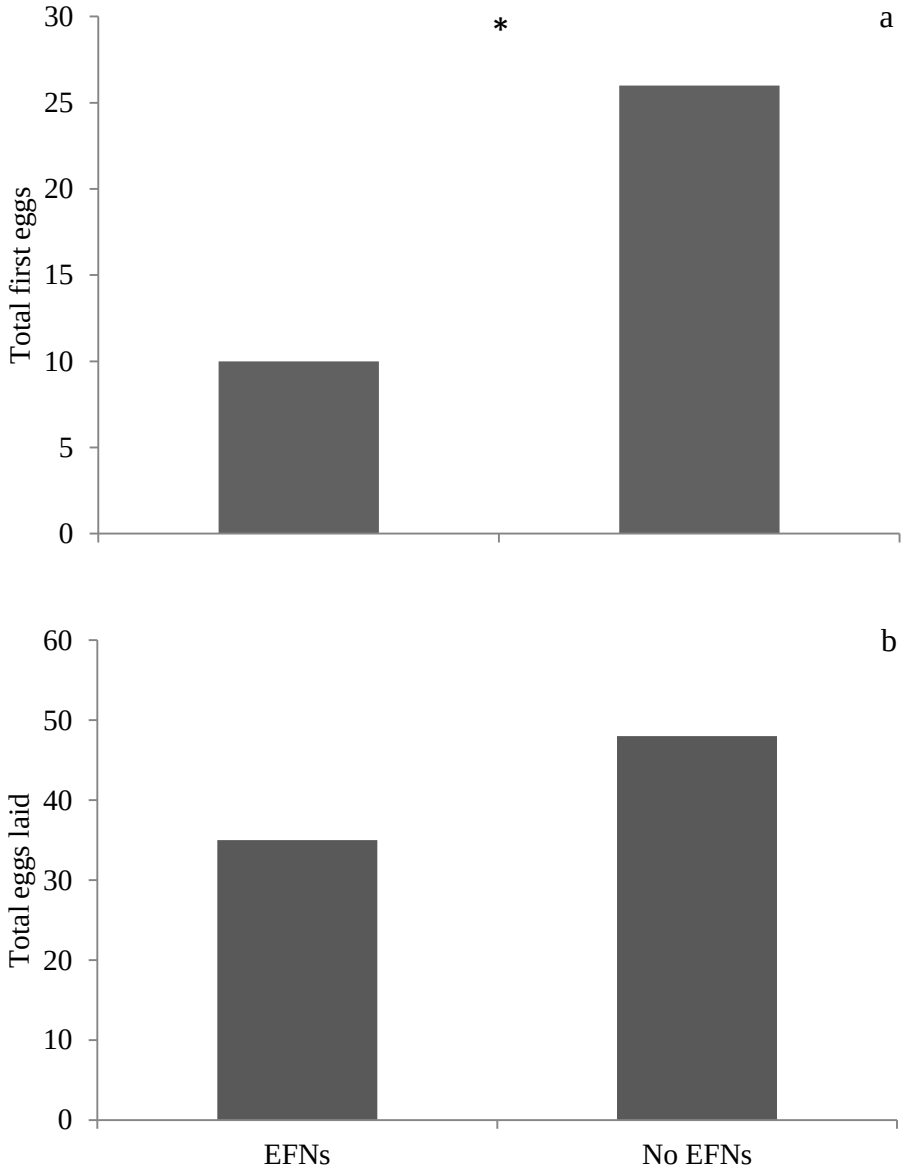

Figure 5. ALM oviposition on leaves with and without EFNs in 2-Way Choice trials. The total number of first eggs (a) and the total of all eggs laid on each leaf (b). Significant differences ($P<0.05$) are represented by asterisks.

Long-Term Survey. Leaf and shoot-level EFN expression had significant but opposite effects on oviposition (Table 2). Leaves without EFNs bore significantly more total eggs, scars, and mines than did EFN leaves ($t = 4.15$, $P < 0.0001$, df = 4290; Figure 6a). In contrast, at the shoot-level, oviposition increased with EFN frequency on both EFN and non-EFN leaves (Figure 7). Leaf tops received more eggs, scars, and mines than did leaf bottoms (Figure 8a). The interactions of leaf-level EFNs and leaf side with shoot EFN frequency were not significant (Table 2). Egg density per leaf surface ($\bar{x} = 1.28$) was highly variable (sd = 3.05) with three or more (up to 20) eggs laid on 14% of the leaf surfaces.

Table 2. Analyses of oviposition (eggs, scars, and mines) and egg removal (scars) in the Long-Term Survey data. Tests of fixed effects from generalized mixed model linear regression for model of eggs, scars & mines.

Model	Source	Den. DF	*F*	*P*
Eggs, Scars, Mines	EFNs	4290	4.16	**0.0413**
	Shoot EFN Freq.	4290	203.29	**< 0.0001**
	Side	4290	112.73	**< 0.0001**
	EFNs*Shoot EFN Freq.	4290	0.52	0.4699
	Side*Shoot EFN Freq.	4290	1.33	0.2493
Scars	EFNs	6	15.56	**0.0076**
	Shoot EFN Freq.	6202	0.04	0.8455
	Side	6	28.48	**0.0018**
	EFNs*Shoot EFN Freq.	6202	0.24	0.6258
	EFNs*Side	6	2.44	0.1696
	Side*Shoot EFN Freq.	6202	3.00	0.0831

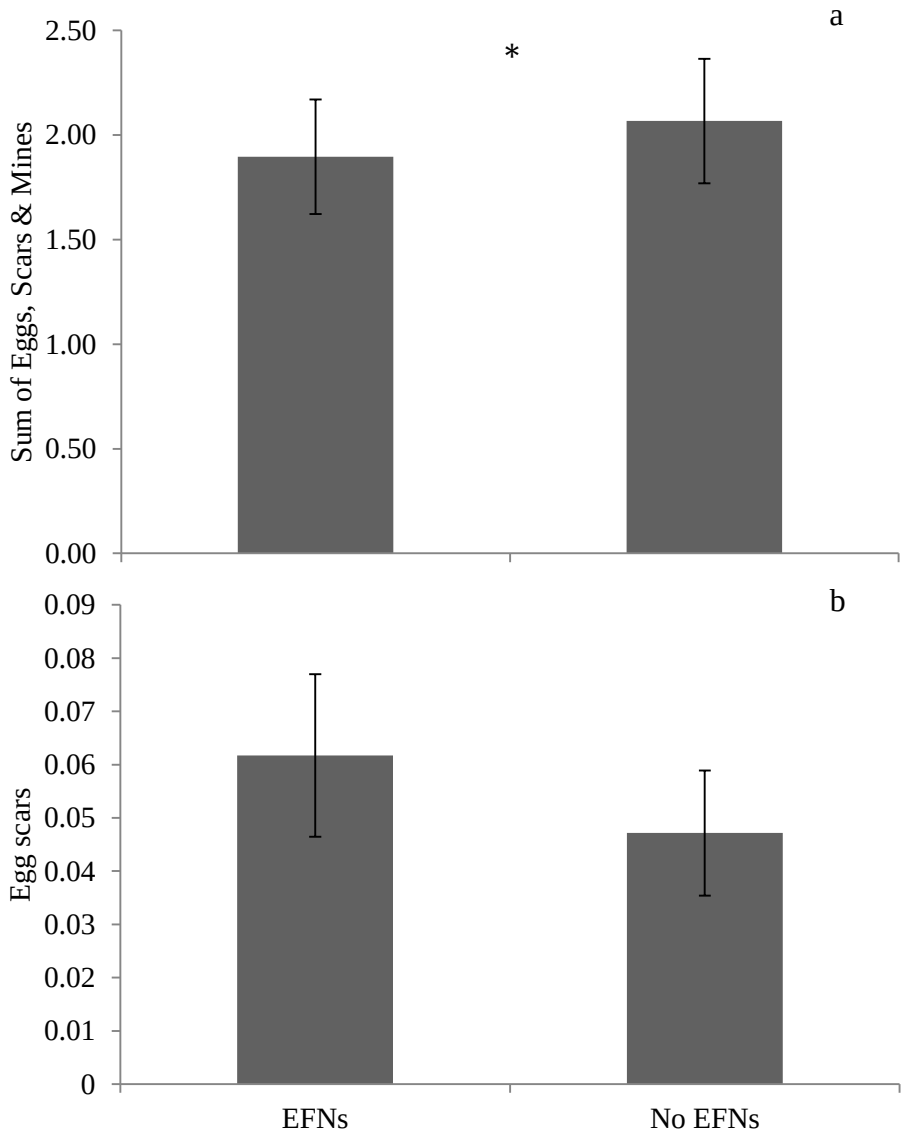

Figure 6. ALM oviposition on leaf types in the Long-Term Survey. Estimated number of eggs, scars, and mines (a), and scars (b) on leaves with and without EFNs. Significant differences in Tukey-adjusted least squares means (P<0.05) are represented by asterisks. Estimates are backtransformed least squares means (± SE) from GLMs.

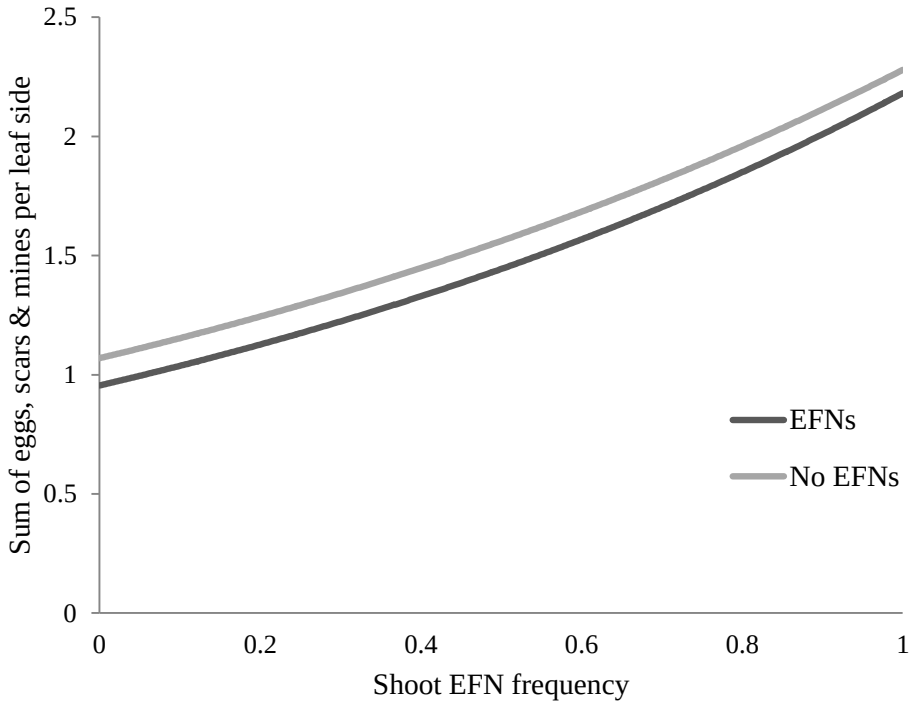

Figure 7. Effect of shoot EFN frequency on the sum of eggs, scars and mines per leaf surface. Estimates are backtransformed from GLMs.

The number of scars per leaf surface was significantly related to EFN expression at the leaf but not the shoot scale (Table 2). More scars were present on leaves with EFNs than without EFNs (Figure 6b). Egg scars on leaf bottoms were more frequent than on leaf tops (Figure 8b). There was no significant effect of shoot EFN frequency or the interactions of shoot EFN frequency and leaf position with EFNs.

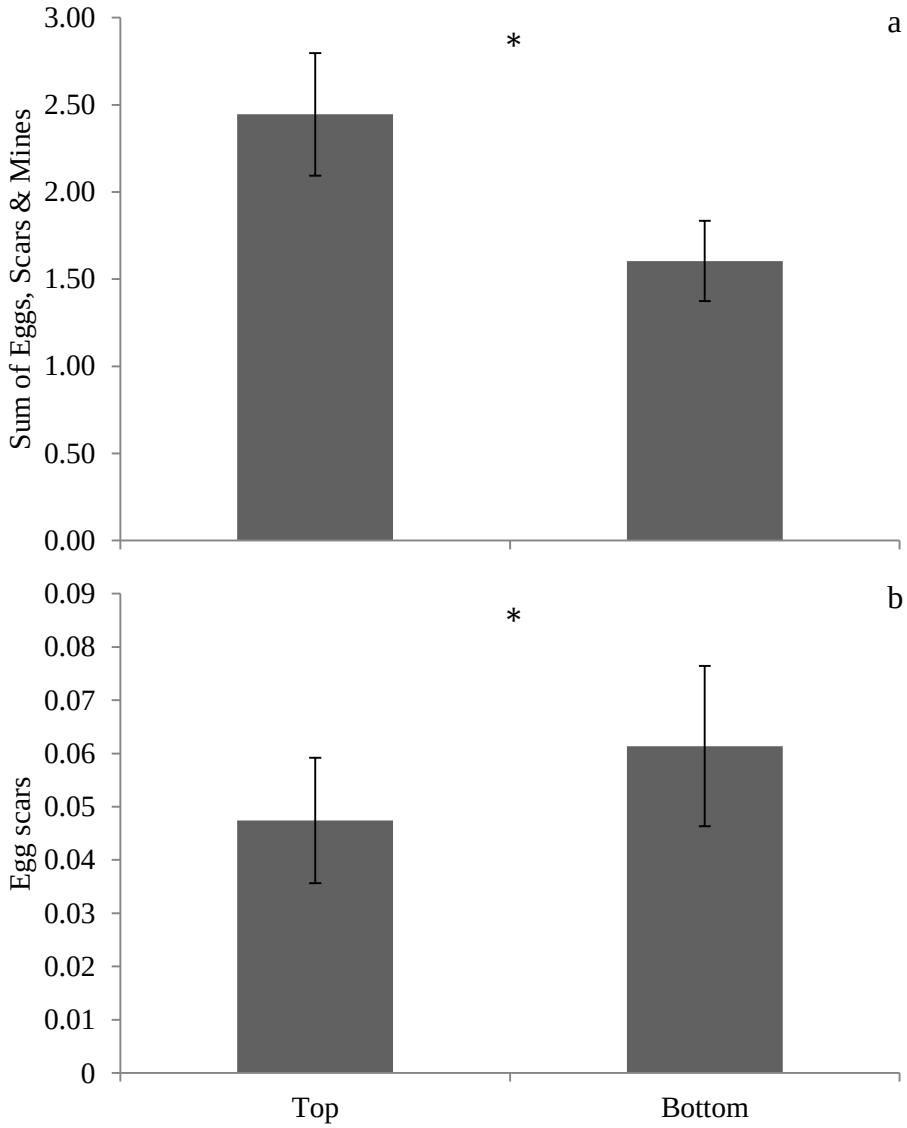

Figure 8. The sum of eggs, scars and mines (a) and scars (b) on leaf tops and leaf bottoms in the Long-Term Survey. Significant difference in least squares means (P<0.05) is represented by an asterisks. Estimates are backtransformed least squares means (± SE) from GLMs.

Egg Tracking Survey. The presence of EFNs interacted with crawling predator exclusion (Table 3) so that significantly fewer eggs were laid on EFN leaves on control ramets (Figure 9). In contrast, oviposition did not differ significantly between leaves with and without EFNs on ramets to which a Tanglefoot® treatment was applied (Table 3, Figure 9). Leaf side interacted with shoot EFN frequency (Table 3) so that least squared mean of eggs on leaf tops was not significantly different than on leaf bottoms at low shoot EFN frequencies (shoot EFN freq. = 0; $t = 0.82$, $P = 0.413$, df = 493), but at high frequencies leaf tops received more eggs than bottoms (shoot EFN freq. = 1; $t = -2.43$, $P = 0.015$, df = 493; Figure 10).

Table 3. Analysis of oviposition (eggs) and egg removal (removals) in the Egg-Tracking Survey. Tests of fixed effects from general linear mixed model regression (Eggs) and logistic regression (Removal).

Model	Source	Den. DF	*F*	*P*
Eggs	EFNs	493	4.13	**0.0426**
	Shoot EFN Freq.	50.75	3.23	0.0784
	Side	493	0.67	0.4129
	Treatment	17.97	1.34	0.2626
	EFNs*Shoot EFN Freq.	346.3	0.66	0.4172
	EFNs*Side	493	3.39	0.0663
	EFNs*Treatment	493	5.26	**0.0222**
	Side*Shoot EFN Freq.	493	6.37	**0.0119**
	Side*Treatment	493	0.29	0.5927
Removal	EFNs	84	4.33	**0.0406**
	Shoot EFN freq.	84	6.01	**0.0163**
	Side	84	0.54	0.4644
	Treatment	84	0.13	0.7229
	EFNs*Shoot EFN freq.	84	0.52	0.4746
	EFNs*Side	84	0.02	0.8832
	EFNs*Treatment	84	0.47	0.4932
	Side*Treatment	84	2.48	0.1190

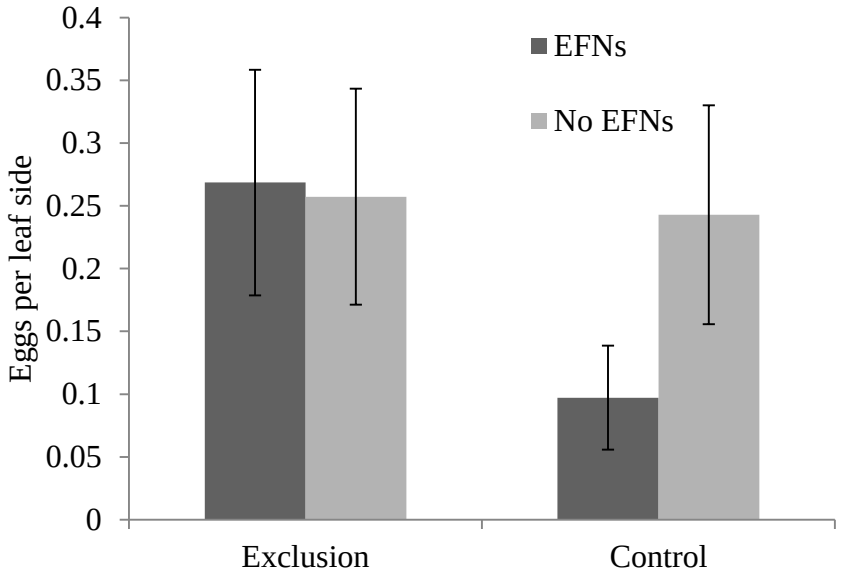

Figure 9. Oviposition on leaves with and without EFNs on ramets with crawling predators excluded versus control. Significant differences ($P<0.05$) represented by asterisk. Estimates are backtransformed least squares means (± SE) from GLMs.

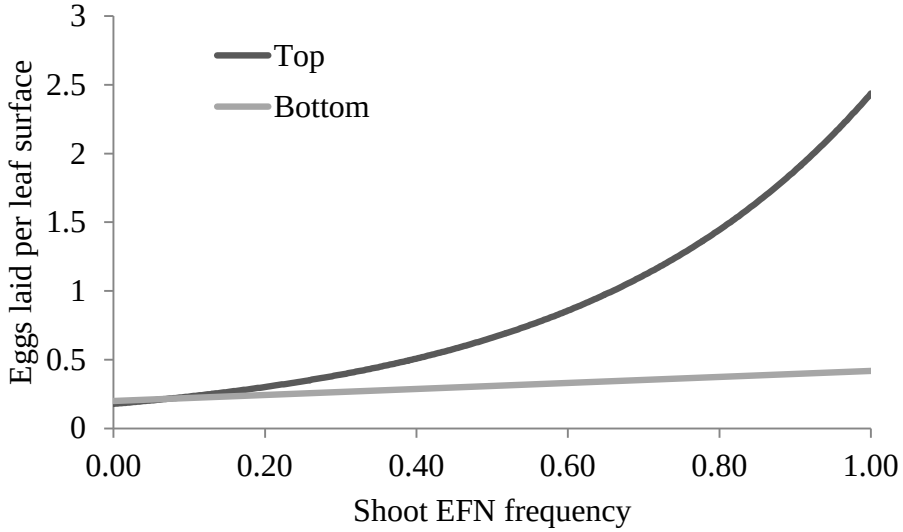

Figure 10. Oviposition on leaf tops and bottoms at varying shoot EFN frequencies. Estimates are backtransformed from GLMs.

Of the 961 eggs laid on leaves within the survey, 33 (3.4%) were removed. Of these, 29 resulted in egg scars. EFN expression at both the leaf and shoot levels significantly affected egg removal (Table 3). More egg removals occurred on leaves with EFNs than on leaves without (Figure 11), and egg removal significantly decreased with increasing shoot EFN frequencies (Figure 12).

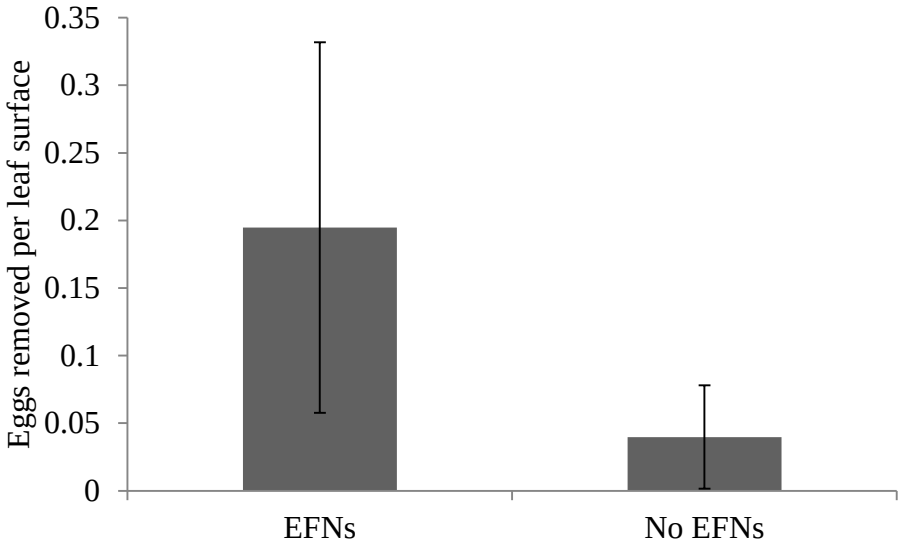

Figure 11. Egg removals on leaves with and without EFNs in the Egg Tracking Survey. Significant difference in Tukey-adjusted least squares means (P<0.05) is represented by an asterisks. Estimates are backtransformed least squares means (± SE) from GLMs.

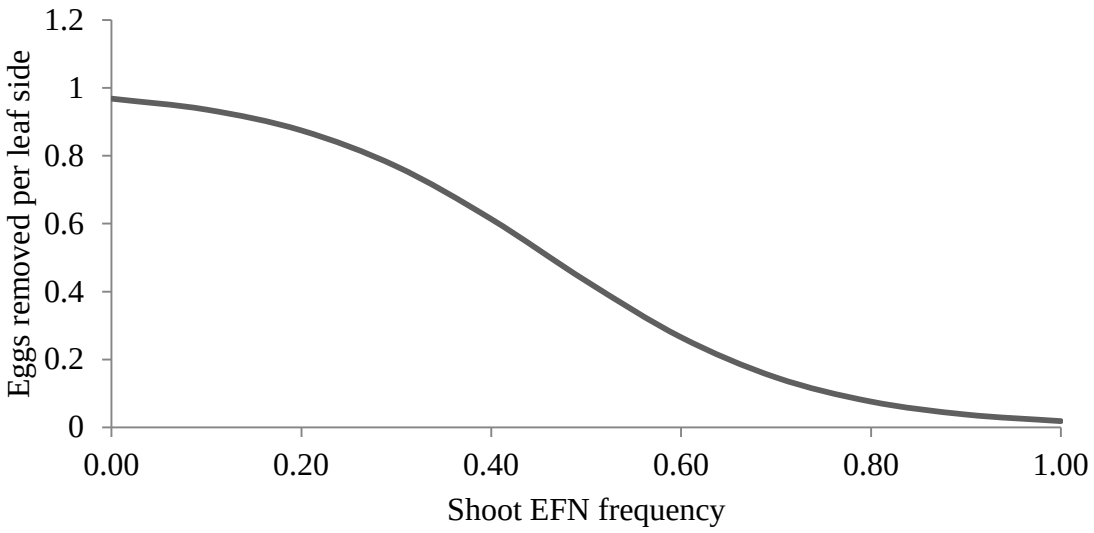

Figure 12. Egg removals at varying shoot EFN frequencies in the Egg Tracking Survey. Estimates were backtransformed from GLMs.

DISCUSSION

We hypothesized that optimal oviposition would have a stronger impact than optimal foraging on oviposition site choice in aspen leaf miners. We expected to find a preference for leaves without EFNs and shoots with low EFN frequency. However, if foraging opportunities influence shoot-level preference, leaf-level choices based on optimal oviposition could be influenced by nearby foraging opportunities. In the present study, oviposition preference for leaves without EFNs supports optimal oviposition while preference for shoots with high EFN frequencies suggests that optimal foraging has a greater role at the shoot scale. In addition, a strong preference for leaf tops was evident. The effects of these preferences on ALM egg distribution are likely to be diluted by intraspecific competition on leaves with multiple eggs, which were common during the ALM outbreak when these surveys were performed. In addition to the influence of EFNs on oviposition, EFN expression influences egg predation, possibly by attracting crawling predators.

EFN-mediated oviposition site preference and egg predation. If EFN leaves attract predators and parasitoids of ALM, females might increase the likelihood of offspring survival by ovipositing on leaves lacking EFNs. On the other hand, if oviposition site selection can be predicted by adult fecundity, and if adult foraging has a significant impact on reproductive output, optimal foraging may be prioritized. Females receive spermatophores during mating and typically mate multiple times (unpublished data). Adult ALM consume nectar at aspen EFNs. While we have not confirmed the sex

of nectaring adults, it is likely that females acquire some resources from aspen EF nectar, thus EFNs may influence female movements and oviposition sites. In the absence of conspecific eggs and predators in two-way choice trials, females preferred to lay their first eggs on non-EFN leaves. Oviposition preference for non-EFN leaves was also evident over seven years of an extensive Long-Term Survey at four sites where shoot EFN frequency and the presence of predators and conspecific eggs were not controlled. Selection for EFN avoidance in ALM oviposition is consistent with high predation on leaves with EFNs relative to leaves without EFNs, and is consistent with the optimal oviposition hypothesis. This evidence suggests that oviposition site preference for non-EFN leaves is selected for through elevated risk of predation on EFN leaves that can be identified by ovipositing ALM.

In addition the role that EFNs play in indirect defense via ants and other predators, they might also reveal leaf quality to ovipositing ALM. Leaves with EFNs are likely to be of lesser quality with regard to ALM larval development than non-EFN leaves. Phenolic glycosides are secondary leaf compounds that provide direct defense against some insect herbivores (Lindroth and Scriber 1988, Lindroth, 2001, Donaldson et al. 2006, Osier and Lindroth 2006, Donaldson and Lindroth, 2007), and in aspen are positively associated with leaf-level EFN expression (Young et al. 2010). Though the effects of secondary leaf compounds on ALM development rate and pupal size are unknown, phenolic glycosides in aspen prolong development time and result in reduced size and fecundity of gypsy moths (Osier et al. 2000). It is possible that preference for non-EFN leaves is driven by the correlation of EFN expression and secondary leaf

compounds. However, oviposition responses in our three-way choice trials did not separate the effects of EFNs, phenolics, and other unknown associated leaf traits. Preference for non-EFN leaves evidenced in the 2-way trials was not found in the 3-way experiment, and EFN occlusion with glue did not stimulate preference. In a related field experiment more eggs were laid on non-EFN compared to EFN leaves regardless of occlusion suggesting that preference for non-EFN leaves is based on EFN-associated leaf traits rather than the presence of EFNs (Mortensen et al. 2013). In our trials, limited air movement within the choice chambers might have exacerbated any negative effects of the glue treatment by interfering with chemoreception of EFN-associated leaf traits.

Optimal oviposition at the leaf scale evidenced by preference for leaves without EFNs is contrasted by the effects of EFN expression on ALM oviposition at the shoot scale. Increasing oviposition with increasing shoot EFN frequency in the Long-Term Survey suggests optimal foraging. Females seem to prefer these EFN-rich neighborhoods to deposit their eggs on non-EFN leaves possibly prioritizing foraging on shoots with abundant sources of nectar, then searching for the best leaves for oviposition. This compromise between optimal foraging and optimal oviposition at leaf and shoot scales partially agrees with Mortensen et al. (2013), who found that preference for non-EFN leaves at low ramet EFN frequencies weakened as EFN frequency increased, ceasing at high EFN frequencies.

By choosing neighborhoods with high EFN frequencies and ovipositing on non-EFN leaves in close proximity to foraging sites females could save time and energy otherwise devoted to seeking out shoots on which non-EFN leaves are relatively

abundant; however this might result in oviposition in neighborhoods of lower quality for larval survival and development. If aspen shoots with high EFN frequencies are analogous to dense foraging patches, and leaves without EFNs are optimal oviposition sites, our field surveys suggest that moths make choices at the shoot scale based on foraging potential before choosing oviposition sites at the leaf scale in accordance with optimal oviposition.

Shoot-level EFN preference in ALM might relate to active periods limited by cool mornings and evenings. Adult ALM are idle during the hot and cold periods of the day and night respectively (personal observation), possibly leading to tradeoffs in ALM oviposition and foraging behavior. If foraging for EF nectar is time consuming or energetically expensive relative to oviposition, adult ALM could better exploit rich foraging patches by ovipositing conveniently at preferred oviposition sites (non-EFN leaves) on shoots with high EFN frequencies.

Evidence of optimality tradeoffs in oviposition (*see* Scheirs and Debruyn 2002a, Scheirs et al. 2004) is accumulating in the literature and could explain the growing number of studies that report no correlation between oviposition site choice and offspring performance (*reviewed in* Mayhew 1997, Scheirs and DeBruyn 2002a). Adult fitness is theoretically maximized when individuals lay high quality eggs on high quality hosts, balancing adult feeding (optimal foraging) and good host choice (optimal oviposition). A number of studies on insect oviposition include examinations of the optimal oviposition hypothesis (*reviewed in* Thompson and Pellmyr 1991, Fujiyama and Katakura 2001; *see also* Craig et al. 1989, Wise and Weinberg 2002, Jallow and Zalucki 2003, Morrison and

Quiring 2009), and linkages between oviposition preference and larval performance support choice adapted to optimize larval survival in many, but not all cases. Recent integration of optimal foraging and optimal oviposition theories (*reviewed in* Scheirs and Debruyn 2002a) highlights cases in which the selection of high quality hosts for larval performance does not explain patterns in oviposition behavior.

Surprisingly, egg removals occurred more frequently at low shoot EFN frequencies in our Egg Tracking Survey. This survey was limited to two sites and 20 ramets, and its findings do not agree with conclusions drawn from more robust surveys. Across nine similar field sites, Mortensen et al. (2011) found that EFN expression is unrelated to egg removal at the leaf scale, but positively related at the ramet scale. These incongruities might be explained by differences in mechanisms of EFN detection in ALM and their predators at leaf, shoot, and ramet scales. Responses to EFN detection in predaceous ants, for instance, might tune in to larger scales of EFN expression than in ALM. For instance, crawling predators such as ants are limited in mobility compared to their winged ALM prey, and might benefit from foraging on ramets with high EFN frequencies whereas ALM can choose among shoots on neighboring ramets. Results from Mortensen et al. (2011, 2013) and this study, though at odds in some respects, nonetheless provide evidence that ALM and their predators make EFN-mediated choices at the leaf scale and at a larger shoot and/or ramet scale.

Intraspecific Competition. Our surveys and related studies consistently find preference for non-EFN leaves in the field where mean conspecific egg densities varied

from 0.3 to 8.5 eggs per leaf surface and can be as high as 82 eggs per leaf surface. Though we found preference for non-EFN leaves in the deposition of the first egg in each 2-way choice trial, we found no preference when considering the total number of eggs. However, in trials with multiple eggs oviposition occurred on both leaves more frequently than on one leaf, suggesting oviposition avoidance of previously laid eggs. Larval interference competition is often intense among mining insects (Quiring and McNeil 1984, Auerbach and Simberloff 1989, Craig et al. 2000) because larvae are limited in foraging area. Larval competition potentially selects for female optimization of larval performance by avoiding sites with relatively high densities of conspecific eggs. In herbivorous insects, uniform egg distribution is expected to result from conspecific egg avoidance (Sugiura et al. 2007). However, strong oviposition site preference resulted in greater oviposition at high quality oviposition sites despite the increased risk of competition on non-EFN leaves.

ALM larvae appear to experience strong intraspecific competition. The single epidermal layer to which they are restricted by their forward-facing mouthparts limits their ability to avoid encounters with conspecifics (Condrashoff 1964). Doak and Wagner (unpublished data) have found a strong negative effect of larval density per leaf surface on ALM survival probability. If avoidance of conspecific eggs weakens preference for other leaf characteristics, effects of competition are expected to dilute the effects of preference for non-EFN leaves that we observed in the choice trials. Preference for non-EFN leaves in the field must therefore be strong enough to overcome this dilution effect.

Leaf side. In Long-Term Surveys, leaf tops were preferred oviposition sites over leaf bottoms. This finding is supported by increased mining damage on leaf tops in previous studies of ALM (Doak et al. 2007, Mortensen et al. 2011). Preference for leaf tops is somewhat surprising since the bottom of each leaf is available to oviposition before the upper surface is exposed, and might be explained by the presence of crawling predators. Predation on leaf bottoms was high relative to leaf tops in Long-Term Surveys, and is reflected by evidence of greater negative effects of predator presence on leaf bottoms in a previous study (Mortensen et al. 2011). Increased predation pressure on leaf bottoms could select for oviposition preference for leaf tops. No evidence of differential predation on leaf tops and bottoms was found in our Egg Tracking Survey, possible due to small sample size.

In addition to implications of EFN expression for optimal foraging on leaf tops, the effects of differing leaf characteristics and microclimates on leaf tops and bottoms might explain the preference for the upper leaf surface. Elevated temperatures associated with sunlight on leaf tops could enhance ALM larval performance by accelerating development, and therefore promote preference for leaf tops. Williams et al. (2003) observed substantially reduced development time in gypsy moth larva at 3.5°C above ambient temperature, and Johns and Hughes (2002) report similar results for the leaf-miner *Dialectica scalariella* Zeller.

Increased oviposition on leaf tops at high EFN frequencies in our Egg Tracking Survey suggests that ovipositing ALM are drawn to leaf tops even during leaf-out when EFNs are small and obscured by furling of the upper surfaces of young leaves. Together

with the lack of any effect of glue in Mortensen et al. (2013), these findings suggest that EFN detection in ALM is based on EFN-associated leaf traits rather than nectar availability.

Predator Exclusion. The exclusion of crawling predators in our Egg Tracking Survey had no effect on predation. However, higher egg predation on leaves with EFNs than on leaves without EFNs suggests that some predators had access to leaves on exclusion ramets. The exclusion treatment used in these studies was previously reported to reduce the presence of ants and *Balaustium* mites, but not spiders resulting in compensatory predation in the absence of ants (Mortensen et al. 2013). The exclusion of crawling predators in the Egg Tracking Survey is likely to have produced the same compensatory effect.

Conclusion. The results of this study suggest possible strategies for ALM oviposition based on EFN expression at leaf and shoot scales. Oviposition preference for leaves without EFNs in our two-way choice experiment points to oviposition avoidance of EFN leaves. This pattern supports optimal oviposition at the leaf level that according to Mortensen et al. (2013) is likely to be mediated by EFN-associated leaf traits rather than EFN expression directly, though our 3-way choice experiment failed to confirm the separation of EFNs and EFN-associated leaf traits. Preference for non-EFN leaves persists in the field independently of shoot-level EFN frequencies. The combination of leaf-level preference for non-EFN leaves and shoot-level preference for high EFN

frequencies suggests an oviposition strategy that optimizes both oviposition and foraging. We have also shown that EFN-mediated predation may drive ALM oviposition avoidance of EFN leaves through increased risk of egg predation on EFN leaves.

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